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(54) Immobilised polynucleotides

(57) Nucleotides can be linked from a 5'-phosphate residue to a support via a dithio (–S–S–) linkage. Polynucleotides thus immobilised can be used in the polymerase chain reaction (PCR) at which a temperature of 90°C is typically required. The –S–S– linkage can be produced by reaction of a thiolated support with a 5'-(thiol–terminated) polynucleotide. For ease of hybridisation of the immbolised polynucleotide, the sequence to be hybridised should be adequately spaced from the support. The invention is useful in any situation in which a polynucleotide such as DNA is required to be separated as a solid phase from a liquid phase, especially in heterogeneous assays and to isolate products of the PCR.
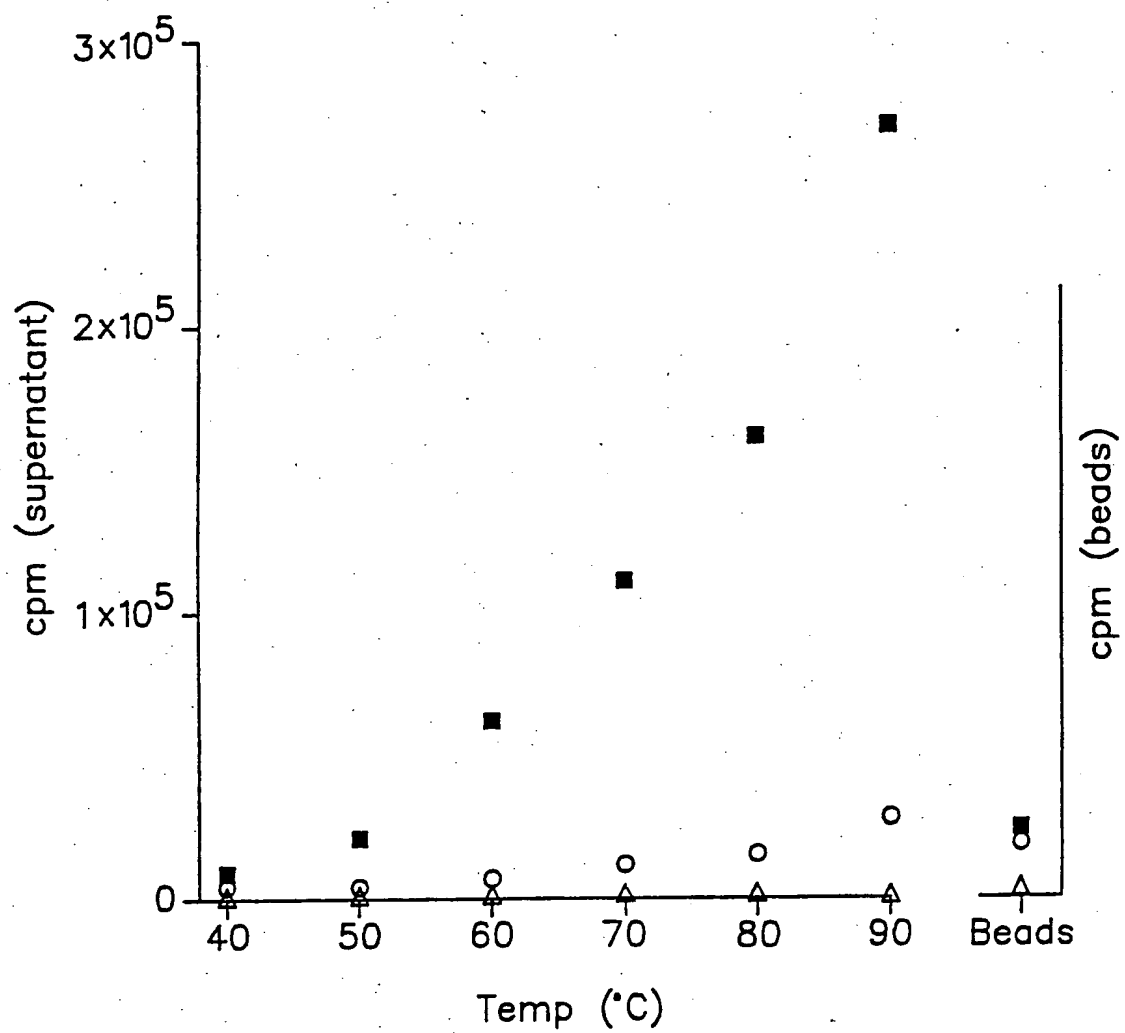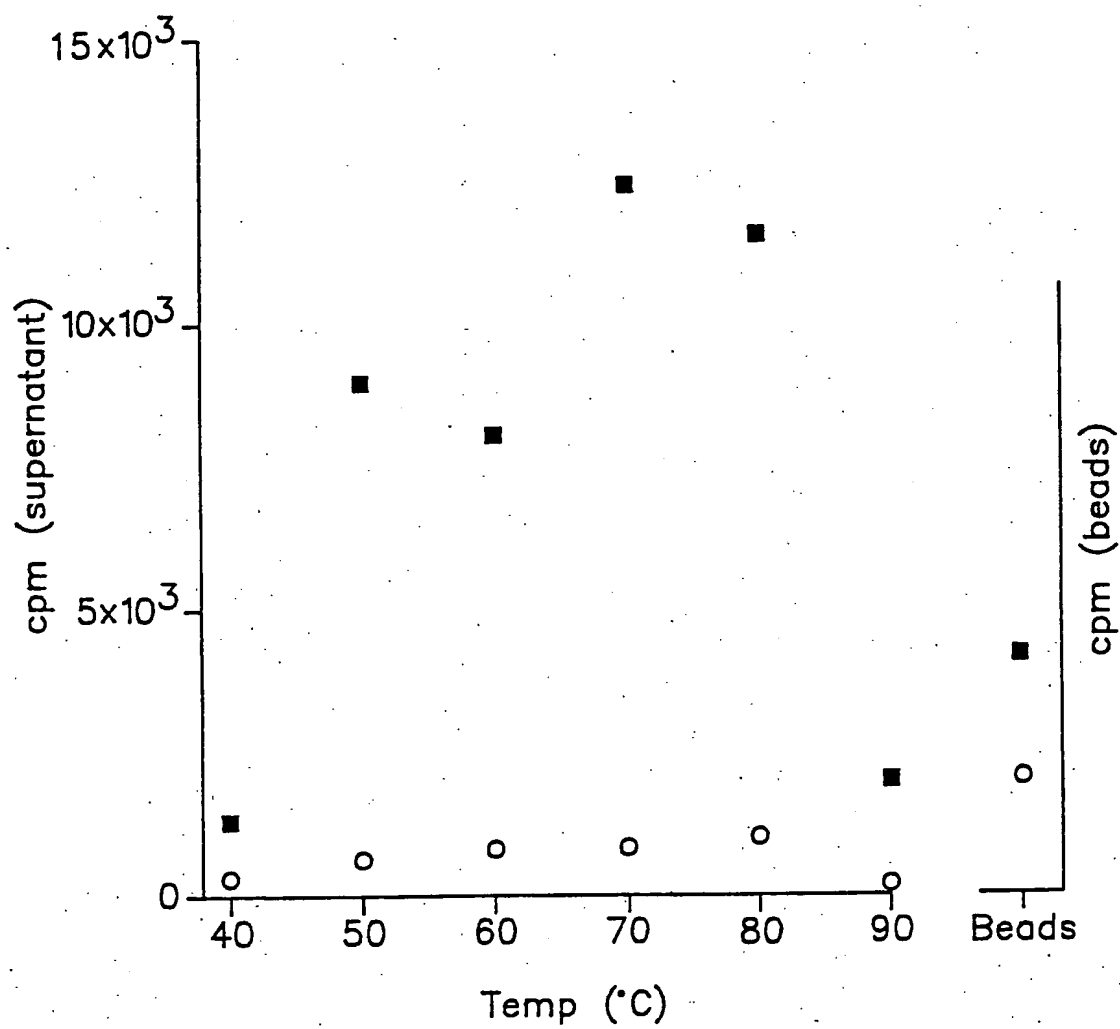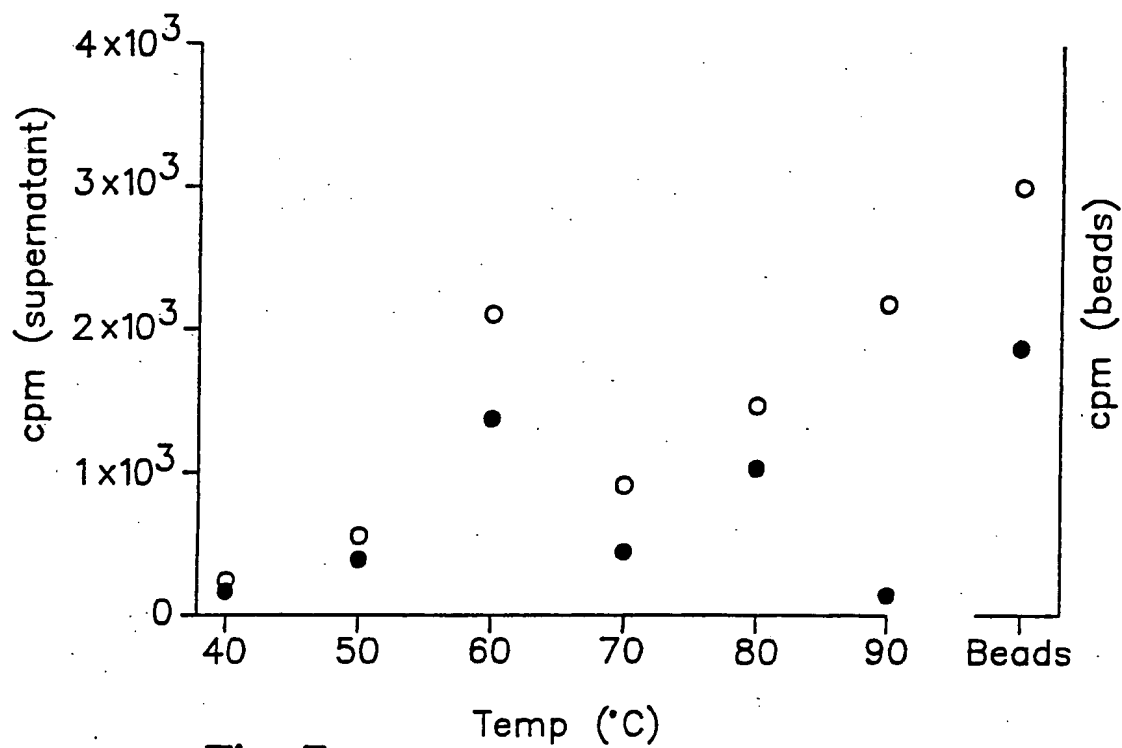
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Fig.1

Fig.2

Fig.3

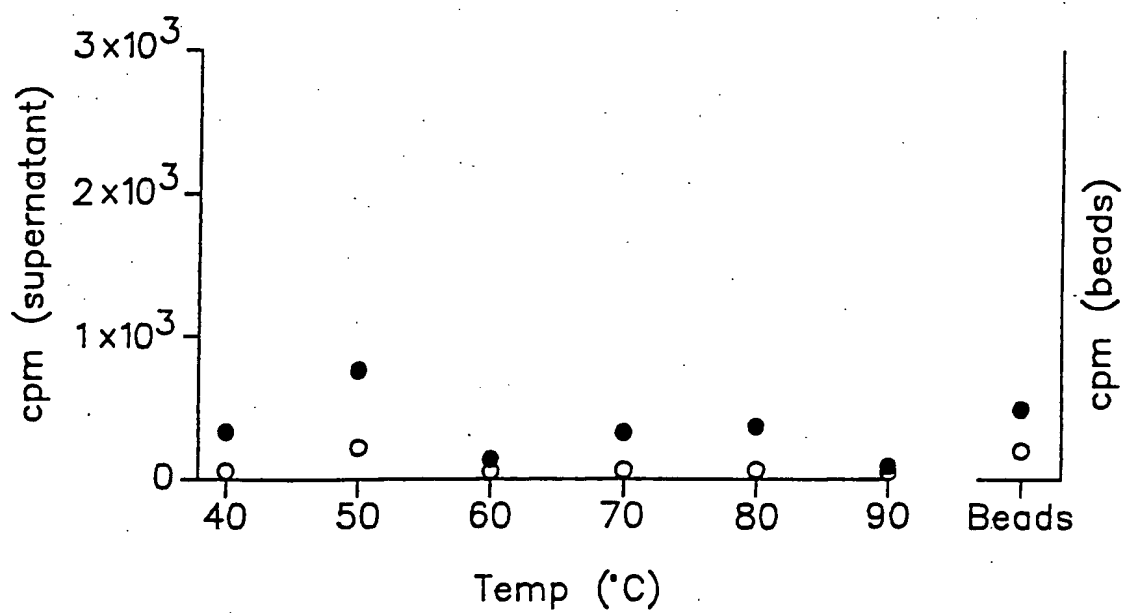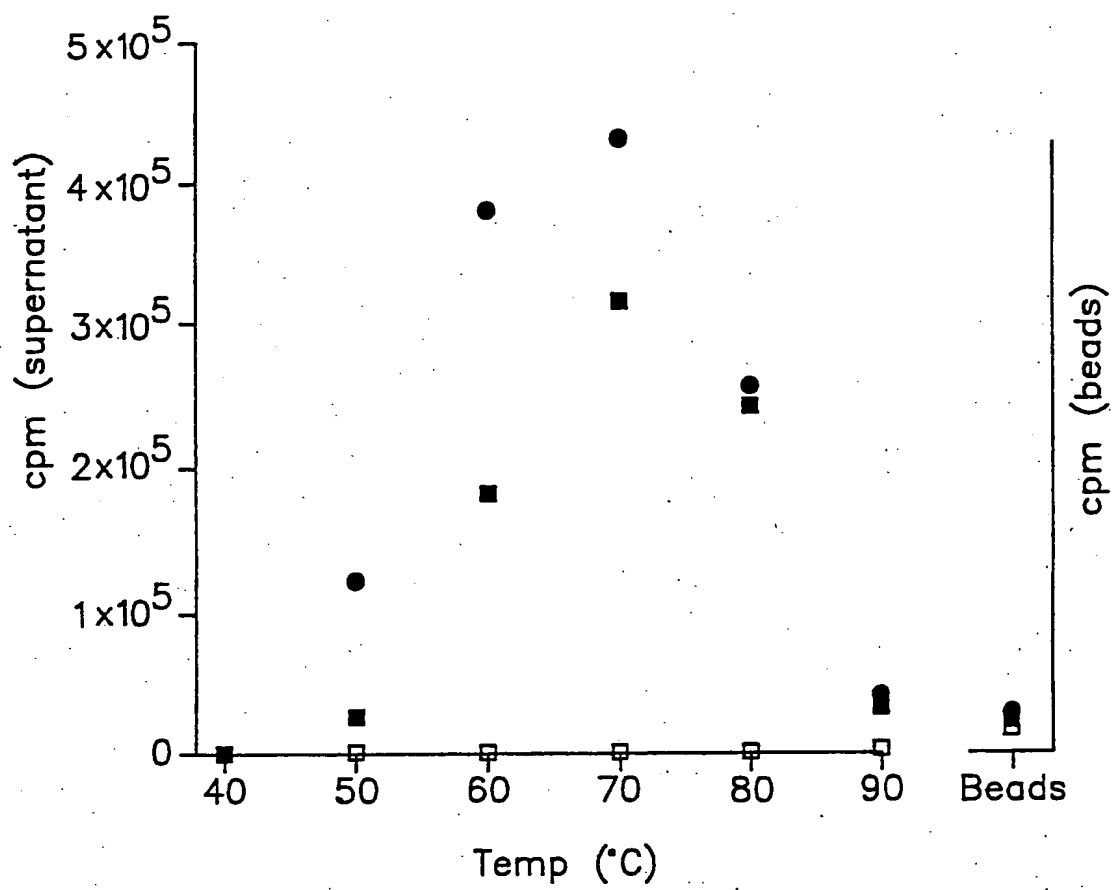Fig.4

Fig.5

IMMOBILISED POLYNUCLEOTIDES

Background to the invention

1. Field of the invention

This invention is in the field of assays for polynucleotides, principally DNA, and relates to the immobilisation of polynucleotides on a support material.

05 #### 2. Description of the prior art

The polymerase chain reaction (PCR), see U.S. Patent 4,683,202 (Mullis), R.K. Saiki et al., Science 230, 130-154 (1985) and H.A. Erlich et al, Nature 331, 461-462 (1988), allows a nucleic acid sequence to be amplified. As applied to DNA, the

10 process comprises the steps of denaturing a duplex to produce two single-stranded (ss) molecules, synthesising complementary DNA to each ss DNA by oligonucleotide priming synthesis, thereby producing a duplex from each ss DNA molecule, denaturing the duplex to produce two ss DNA molecules (one of the original

15 length, one of length governed by the position at which priming began), using each of these molecules as the template for a further primed synthesis and so on for as many cycles as desired. In view of the use of heat to denature the DNA, the DNA polymerase enzyme used for the primed synthesis is preferably one

20 which is stable at the denaturing temperature, in order to avoid addition of fresh polymerase at each cycle. The use of a heat-stable polymerase from Thermus aquaticus enables the PCR amplification of DNA (directly from DNA, or indirectly from RNA) to be carried out at temperatures ranging from 30 to 100°C, at

25 various stages of the process and dependent on the nature of the sequence to be amplified, in automated equipment.

The PCR is potentially of immense value in diagnostics since it enables either the analyte nucleic acid or the duplex produced upon hybridisation of the analyte to a labelled probe to be

30 amplified and thereby very small amounts of nucleic acid to be detected. An amplification of 10^{20} within 4-6 hours is possible.

Hybridisation of native and/or synthetic DNA to complementary DNA/RNA sequences is critical to the performance of PCR and many other DNA/RNA assays. Detection of analyte sequences with or without amplification is also dependent on hybridisation of

05   complementary sequences to a labelled probe. Most of these present techniques require immobilisation of the analyte or probe sequences (e.g. to nylon membranes) prior to hybridisation. Consequently the time and complexity of the protocols increases whilst non-specific linkage employed reduces the amount of

10   specific analyte or probe available for hybridisation. Separation and concentration of amplified analyte sequences by solution hybridisation following PCR is highly desirable to readily enable detection and increase sensitivity (and quantitation) and decrease incubation times by favouring the

15   kinetics.

The linkage of oligonucleotides to solid phase particles for collection, separation and detection of analyte sequences by solution hybridisation has been investigated by several groups [see review by J.A. Matthews and L.J. Kricka, Anal. Biochem. 169,

20   1-25 (1988)]. Matrices commonly used have been control pore glass [S.S. Ghosh and G.F. Musso, Nucleic Acid Res. 15, 5353-5390 (1987)], latex [S.F. Wolf et al., Nucleic Acid Res. 15, 2911-2926 (1987)], polystyrene [Y. Nagata et al., FEBS Lett. 183, 379-382 (1985)], cellulose [T. Goldkorn and D.J. Prockop, Nucleic Acids

25   Res. 14, 9171-9191 (1986)] and magnetically attractable beads of cellulose [J.A. Langdale and A.D.B. Malcolm, Biochem. Soc. Trans. 12, 693-694 (1984) and P.J. Nicholls, J.A. Langdale and A.D.B. Malcolm, Biochem. Soc. Trans. 15, 140 (1987)] or styrene polymer [V. Lund et al., Nucleic Acids Res. 16, 10861-10880 (1988)].

30   Several chemistries have been applied to the attachment of the oligonucleotides, based on linkage via carboxy or amino groups (e.g. using carbodiimide or cyanogen bromide or tosyl-activated beads) [J.A. Langdale and A.D.B. Malcolm, Biochem. Soc. Trans. 12, 693-694 (1984), R. Polsky-Cynkin et al.,Clin. Chem. 31,

35   1438-1443 (1985), J.A. Langdale and A.D.B. Malcolm, Gene 38,

201-210 (1985), V. Lund et al., Nucleic Acids Res. 16, 10861-10880 (1988), P.J. Nicholls, J.A. Langdale and A.D.B. Malcolm, Biochem. Soc. Trans. 15, 140 (1987), L. Clerici et al., Nucleic Acid Res. 6, 247-257 (1979), D. Rickwood Biochem.
05 Biophys. Acta 240, 515-521 (1973), H. Bunemann, P. Westhof and R.G. Herrmann, Nucleic Acid Res. 10, 7163-7181 (1982) and A. Jungell-Nortamo et al., Molecular and Cellular Probes 2, 281-288 (1988)] or introduction of electrophilic groups at the 5' end of the oligo and then reacting the oligo with a nucleophilic
10 residue on the support [J.N. Kremsky et al., Nucleic Acid Res. 15, 2891-2909 (1987)]. Specific attachment of oligonucleotides has been achieved to varying degrees by all methods (generally of 10-20 µg or 0.04-1.0 nM oligonucleotide per mg of beads). However, the availability of amino and carboxyl groups on the
15 nucleotide side arms resulted in poor specific coupling via the 5' end of the oligonucleotide. Consequently, only a small proportion of the attached oligonucleotide is available for hybridisation (typically femtomole amounts of analyte per mg of beads) [S.S. Ghosh and G.F. Musso, Nucleic Acid Res. 15,
20 5353-5390 (1987)].

In the Goldkorn and Prockop paper, oligo-dT cellulose is linked to an oligonucleotide via a polynucleotide chain by the steps of (1) poly dA-tailing the 3'-ends of a double-stranded oligo, (2) hybridising the poly dA-tailed DNA to the oligo-dT
25 cellulose, (3) filling in the gap between the last dT of the oligo-dT cellulose and the 5'-end nucleotide complementary to the 3'-nucleotide onto which the poly A tail was added, by the T4 ligase/Klenow fragment method and (4) separating by heat denaturation the filled-in strand, now attached to the oligo dT
30 cellulose, from the other strand, which is attached to the oligo-dT cellulose only indirectly, via its complementary strand, as a result of the dA-dT hybridisation. This procedure involves multiple steps and expensive reagents and is exclusive to cellulose, this being the only known support to which a dT chain
35 can readily be linked.

Various other esoteric linkages are mentioned in the Matthews and Kricka review paper, see Table 7 on page 15.

A-C. Syvänen, Nucleic Acids Research 16, 11327-11338 (1988) describe 5'-biotinylating DNA molecules and joining them to
05 avidin-coated polystyrene particles. The particles are used to capture 5'-biotinylated products of the PCR. This methodology cannot be used in the PCR itself because the biotin/avidin interaction is not sufficiently heat-stable.

10 Summary of the invention

It has now been found that nucleotides can be linked to a support via a dithio (-S-S-) linkage, which results in exceptional stability of linkage. In particular, the supported polynucleotide can participate in a PCR reaction carried out at
15 elevated temperature. The dithio linkage is formed by coupling a thiol (-SH) group or a reactive derivative thereof on the polynucleotide with a thiol group or reactive derivative thereof on the support. Further, when a polynucleotide is thus linked, then, provided that the support is sufficiently distanced from
20 the sequence of interest, that sequence can be hybridised strongly to a labelled complementary probe.

Since the dithio linkage is stable at the highest temperature (about 90°C) typically used in the polymerase chain reaction (PCR), polynucleotides thus linked to beads can be used in the
25 PCR. They are also useful for a variety of purposes in heterogeneous assays of nucleic acids.

Accordingly, an important feature of the invention consists in an immobilised polynucleotide material comprising a support bound to a 5'-end of at least one polynucleotide through a
30 linkage which includes a dithio (-S-S-) group. The term "polynucleotide" encompasses DNA, RNA, oligonucleotides and any modified or labelled form of conventional nucleic acid in which pairable bases are arranged in helical chains.

An important subsidiary feature comprises an immobilised
35 polynucleotide material comprising a support bound to a 5'-end of

at least one polynucleotide which contains a "c-target" base
sequence complementary to a "target" base sequence of interest,
said support being bound through a linkage as defined above and
which is effective to distance or space the support from the
05   "c-target" sequence so as to permit the c-target sequence to
hybridise to the target sequence. (References herein to
hybridisation are to be construed to include annealing of primers
to longer sequences as occurs in the PCR).

It has been found that the linkage in this invention results
10   in a particularly high degree of hybridisation, probably because,
unlike the CNBr and carbodiimide methods of attachment normally
used, it is specific to 5'-end attachment. It is believed that
in these conventional methods, there is some reaction between
amino groups on DNA bases and the activated supports. The
15   resultant attachment of supports to mid-chain bases would
interfere with base-pairing and therefore hybridisation.

The invention further provides a method of carrying out a
polymerase chain reaction (PCR) in which an immobilised
polynucleotide material of the invention is substituted for
20   either or both of the oligonucleotide primers used in the PCR and
the amplified product of the PCR is separated on the solid
phase. Thus, as applied to a conventional PCR, the c-target
sequences of two immobilised polynucleotides anneal to respective
target sequences of the polynucleotide to be amplified, one
25   target sequence being at a 5'-end of one strand and the other
being at the 5'-end of the other strand.

In another aspect, the invention includes a method of
heterogeneous assay for an analyte nucleic acid, which comprises
subjecting the immobilised polynucleotide material of the
30   invention, having a c-target sequence, to hybridisation with the
target sequence of analyte nucleic acid present in a liquid phase
analyte, separating the phases and determining the occurrence or
extent of hybridisation. Preferably, the analyte nucleic acid is
labelled either directly or by hybridisation at a second site
35   thereof with a labelled complementary polynucleotide. Such an

assay is preferably used in conjunction with a liquid phase PCR, to detect a product of the PCR. It can also be used in any nucleic acid assay context.

The invention further includes a method of producing an immobilised polynucleotide material of the invention which comprises reacting a 5'-(thiol-terminated) derivative of a polynucleotide with a support having a thiol group thereon, either or both of said thiol groups being optionally derivatised as co-reactive -S-S- linkage-producing derivatives, to form a said -S-S- linkage between said thiol groups. Any chain atoms (a) between support and its pendant thiol group and (b) between 5'- nucleotide phosphate residue and its pendant thiol group are preferably thermally stable at the maximum temperature likely to be essential in the PCR reaction, normally 90°C–100°C.

For most, but not necessarily all, uses of the invention, the support needs to be distanced from the sequences which it is desired to hybridise. Desirably, the spacing is provided wholly by the linkage, that is to say between the 5'-end of the last nucleotide and the support. However, it could be provided partly or wholly by attaching a sequence of irrelevant nucleotides to the last nucleotide of interest and providing the thiol termination on the last of the irrelevant nucleotides. In other words, the spacing between the last nucleotide of interest, usually a c-target nucleotide, and the support can contain nucleotides.

Additional description of prior art

Oligonucleotides having a thiol (SH) group pendant from the 5'-terminus are not, as a class, novel. B.A. Connolly and P. Rider, Nucleic Acids Research 13, 4485–4502 (1985) prepared such compounds by reacting S-trityl-ω-mercaptoalkanols with O-methoxymorpholinochlorophosphate to give S-trityl-O-methoxy-morpholinophosphites of formula

$$Ph_3-S-(CH_2)_x-O-P \overset{\displaystyle OCH_3}{\underset{\displaystyle N}{\big|}}$$
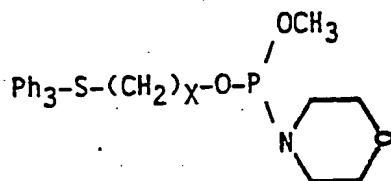
where Ph = phenyl and x = 2, 3 or 6, and then coupling these compounds with oligonucleotides which had been prepared by a solid phase phosphoramidite method, oxidising the resultant phosphite to phosphate, and de-protecting the thiol group.

05 Connolly and Rider used these compounds in reactions to label the thiol-terminated oligonucleotides with the fluorophore, N-iodoacetyl- N'-(5-sulpho-1-naphthyl)ethylenediamine (AEDANS) or N-(1-anilino-4-naphthyl)maleiimide (ANM). In the reaction with AEDANS, the iodine atom is displaced, forming a $-S-CH_2-CO-$

10 linkage, while in the reaction with ANM an electrophilic addition of the double bond produces a linkage:
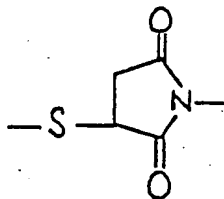
15 In PCT Patent Specification WO 89/02931 (Cetus) published 6th April 1989, oligonucleotide "functionalizing reagents" are described. These have a pendant thiol, amino or hydroxyl group linked to the 5'- phosphate group by a spacer chain of formula $-(CH_2)_2-(OCH_2CH_2)_n-CH_2CHR^*-O-$ where the oxygen atom shown is that

20 of the phosphate group and R^* represents H, CH_2OH or tritylated CH_2OH. These reagents are used for reaction with a horseradish peroxidase (HRP) derivative in order to label the oligonucleotide with an enzyme. The HRP derivative has a maleiimide termination, producing the same kind of linkage as described in the Connolly

25 and Rider paper.

Thiol linkages have been used to attach proteins to supports. See, for example, "Solid Phase Biochemistry" ed. W.H. Scanten, John Wiley & Sons 1983, Chapter 7 "Immobilized Enzymes" by John F. Kennedy and J.M.S. Cabral, pages 301 and 302

30 "Affinity Chromatography - principles and methods", published by Pharmacia AB, 1986-1988, pages 35-45.
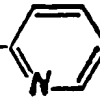
Brief description of the drawings

Figs. 1 and 2 are graphs in which the amount of hybridisation achieved by immobilised polynucleotides of the invention, assayed by heat-denaturing labelled hybrids, is represented by plots of amounts of labelled material released from the hybrids into solution against temperature of denaturation and compared with various controls;

Figures 3 and 4 are similar graphs but referring to immobilised polynucleotides of the prior art; and

Figure 5 is a similar graph showing the effect of the inclusion of a spacer arm in the immobilised polynucleotides of the invention.

Description of the preferred embodiments

While the preferred use of the invention, as currently contemplated, is in the PCR reaction, the invention can be used in the context of any procedure of immobilising or insolubilising a polynucleotide, especially a heterogenous assay procedure. Thus, the invention is applicable in such assay procedures described in the above-mentioned Matthews and Kricka review paper.

The support material is not critical and can be any of those conventionally used. One convenient material is beads which already have thiol groups attached. These are available commercially for the purposes of affinity chromatography of proteins. See, for example, the above-cited "Affinity Chromatography - principles and methods", published by Pharmacia AB, which describes S-protected thiolated "Sepharose" beads of formula:

Sepharose residue $-O-CH_2-CH(OH)-CH_2-S-S-$
(the 2-pyridyl group being protective).

Various glasses can be used as supports when activated to provide suitable functional groups. Thus controlled pore glass (CPG) or any other glass of high surface area can be used.

Virtually any conventional chemical linkages can be used in building up a chain between the support to the -SH termination, even the CNBr or carbodiimide method, so long as they do not

provide the final attachment to the polynucleotide. Obviously, for use in the PCR, thermally susceptible linkages are to be avoided.

05 While the elements of the linkage can be provided in any order, it will be appreciated that it is desirable not to cause any reaction which might be non-specific. It will almost always be desirable to form the -S-S- bond last when preparing the immobilised polynucleotides. By way of example a conventional aminoalkylating agent can be reacted with a support such as CPG

10 to provide amino terminations. The amino-functional support can then be reacted with cysteine in a peptide coupling reaction to provide the SH termination required. However, preliminary indications suggest that such a cysteine link might not produce the desired high specificity of binding of the support to the DNA

15 to be immobilised and at present a mercaptoalkyl termination is preferred. Thus, a support, such as CPG is preferably silanised by an (ω-mercaptoalkyl) trimethoxysilane, conveniently (3-mercaptopropyl) trimethoxysilane. This silanised support is then reacted with the thiol- terminated polynucleotide.

20 The linkage between the polynucleotide and the support will normally include a spacer arm between the polynucleotide and the -S-S- linkage, the -S-S- linkage and the support, or both. Alternatively or additionally the polynucleotide can itself contain a spacer arm, of one or more nucleotides, usually 3 to 20

25 and preferably 4 to 15, between its 5'-end and the nearest "relevant" nucleotide. Where the polynucleotide is a sequence complementary to another sequence to which it is to be hybridised, the "relevant" nucleotide will be the last nucleotide of that complementary sequence, (the "c-target" sequence). This

30 nucleotide spacer arm consists of "irrelevant" polynucleotides which do not interfere with or participate in hybridisation. For the purposes of reckoning its length, the nearest "relevant" nucleotide is not counted in, but the 5'-end nucleotide is counted in. The total length of spacer arm (whether in the

35 linkage or the polynucleotide or both) will depend on the nature

of the linkage and its spatial configuration. In general, a straight chain of from 5 to 27 atoms and nucleotides between the support and the relevant 5'-end nucleotide (inclusive of the two S atoms) is suggested. Too short a chain would tend to bring the

05 support too close to the polynucleotide chain for successful hybridisation to occur, while if the linkage is too lengthy the support might dangle at such a distance as to wrap itself over the relevant 5' end nucleotide and its neighbours. This would probably interfere with successful use of a supported

10 oligonucleotide as a primer in the PCR.

Preferably the total spacer arms conform to the general formula $(N)x-(L_1)y-S-S-(L_2)_z$ e.g. in the manner illustrated below, using arbitrary, imaginary sequences:

```
--NNNGCTTAGCTNNNNNNNNNNNN 3'
     CGAATCGA               5'
              \
              (N)x-(L1)y-S-S-(L2)z-Support
```
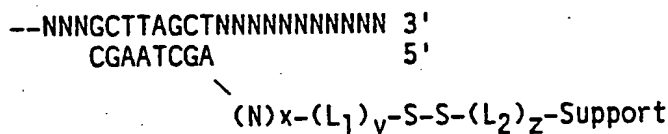
where the top sequence is that of the target (analyte)

15 polynucleotide, the bottom sequence is that of an oligo primer immobilised according to the invention; N represents a nucleotide in the spacer arm upstream of the end of the "c-target" sequence;

L_1 and L_2 represent atoms in a covalent chain from the end

20 nucleotide to the support, on each side of the two sulphur atoms of the -S-S-linkage;

x, y and z are 0 or integers and the sum of x, y and z is 3 to 25, preferably 4 to 25, especially 6 to 20.

It will be appreciated that the invention is usable merely

25 for isolating a polynucleotide without necessarily hybridising or annealing it. In such circumstances a spacer arm will frequently offer no advantage.

The PCR is described in the above-cited references, the disclosures of which are herein incorporated by reference. Any

30 nucleic sequence can be specifically amplified by the PCR. It is only necessary that a sufficient number of bases at each end be

sequenced so that two oligonucleotide ("oligo") primers can be prepared, complementary to sequences at or near the ends of each strand or, at least, sufficiently well separated that the sequence of interest lies between the 3'-ends of the two primers. One oligo primer is called the forward primer, because it primes 5' to 3' synthesis of complement to the coding strand of the polynucleotide. The other is the reverse primer which primes 5' to 3' synthesis of complement to the non-coding strand. If neither strand codes, the designations are arbitrary.

The PCR normally comprises broadly three steps per cycle:

(1) annealing forward and reverse oligonucleotide primers to the strands of a polynucleotide to be amplified, these strands having been first separated, annealing being carried out typically at a temperature of 30 to 60°C;

(2) extending the primed sequences along their respective template strands of the polynucleotide to be amplified, at a temperature typically of 60 to 80°C.

(3) separating the synthesised strands from their templates at a temperature effective for this purpose, typically from 80 to 100°C, especially at about 90°C.

The cycle is then repeated, using the products of each separation step (3) as the templates for the ensuing annealing step (1) of the next cycle. Products are conveniently detected by labelling the nucleotides used for the chain extension and determining the label attached to the products. Labelling can be done radioactively e.g. with ^{32}P or by biotinylation, for example, and the labelled products immobilised and separated from the reaction medium. In another mode, the PCR can be carried out in solution and the products detected by carrying the PCR to the end of a separation step (3), and probing the strands with a probe which is attached to a support or to which a support is later attached. The amount of label attached to the support is then determined.

Of course, it is not critical to label the nucleotides incorporated in the PCR. Instead the PCR products can be

subjected to a sandwich assay in which a two-site probe is used, site A being capable of binding specifically to the PCR product which it is hoped to detect and site B being capable of hybridising with an immobilised polynucleotide of the invention.

05   Another method of detecting PCR products on a solid support depends on measuring the thickness of the layer deposited on the solid phase. This can be done by measuring changes of optical path through the layered film: see literature of Medical Products Inc., Boulder, Colorado, USA.

10   It is also possible to use the 5' thiol-derivatised primers (carrying a free or protected -SH group) in solution in the PCR and subsequently linking the amplified labelled (or unlabelled) products to the thiol-derivatised solid support by means of the invention. The attached PCR products may be detected by any

15   number of methods including those cited above.

Products attached to the solid phase through the linkage of the invention can be released therefrom by any of the known methods of breaking -S-S- bonds, such as by prolonged action of a high concentration of dithiothreitol or 2-mercaptoethanol. Note,

20   however, that the relatively low concentrations of these reagents often present in conventional PCR buffer systems will not normally break the S-S bonds.

Any number of polynucleotides can be linked to the same integral unit (particle, sheet, bead, tube etc.) of the support

25   material, provided that there is no interference between polynucleotides which would hinder their end use, e.g. in an assay.

The following Examples illustrate the invention.

EXAMPLE 1

30   Example 1 illustrates the hybridisation capacity of an oligonucleotide bonded to a support via an -S-S- linkage, in accordance with the invention. The oligonucleotide was used with and without a 4-nucleotide spacer between the c-target sequence and the 5'-end.

35   All operations were carried out at room temperature (about 20°C) except as otherwise described.

THE OLIGONUCLEOTIDES AND PROBES USED

The oligonucleotide synthesised for coupling to the beads is that of the forward primer complementary to a sequence of the human papilloma virus type 16 successfully applied in the PCR for
05 amplification of a 120 base-pair fragment of HPV16 DNA:

5' TCAAAAGCCACTGTGTCCTG 3' (20mer) D99 (in-house designation) identified as Sequence ID No. 1.

(1) To prepare a thiol-terminated oligo, a 5'-spacer was added, composed of an additional 4 dTTP residues, the last 5' terminal
10 dTTP being a fully protected thiol phosphoamidite.

(2) For comparative purposes, the same oligo as in (1) above was prepared, except that an "aminolink II" was incorporated on the terminal 5' dTTP (4 dTTP spacer included). "Aminolink II" is a reagent sold by Applied Biosystems Inc. for derivatisation of the
15 5'- terminus of a synthetic oligonucleotide. It is a methyl phosphoramidite of formula $CF_3CO\ NH(CH_2)_6OP(OCH_3)N[CH(CH_3)_2]_2$ and is used in the beta-cyanoethyl phosphoamidite synthesis to introduce an aminohexyl termination (the terminal CF_3CO- group being cleaved off at the end of the synthesis).

20 (3) The hybridisation probes prepared were either a sequence complementary to D99 i.e:

3' AGTTTTCGGTGACACAGGAC 5' (anti-D99) identified as Sequence ID No. 2.

or, as a control, the reverse primer sequence oligo for HPV16 i.e:
25 3' ACGTCTAGTAGTTCTTGTGC 5' (non-complementary to D99 oligo) identified as Sequence ID No. 3.

SYNTHESIS OF OLIGONUCLEOTIDES

The oligos were synthesised on a Biotech Instruments BT8500 automatic machine, using the well established cyanoethyl
30 phosphoamidite coupling chemistry. The last base to be added at the 5' end of the oligo was a fully dimethoxytrityl protected, thiol phosphoamidite, introduced via line Q on the BT8500. The oligo was cleaved from the controlled pore glass solid phase in the normal way with aqueous ammonia, using a 'trityl ON' cleavage
35 method. The free oligo was then dried at 50°C under vacuum. It is stable for long periods at -20°C.

COUPLING OF THIOL-TERMINATED OLIGONUCLEOTIDE TO BEADS VIA DITHIO LINKAGES.

(i) activation of thiol oligo:

0.04 µmole of the oligo, (10 OD_{260} units) was dissolved in
05 100 µl of 0.1M triethanolamine acetate (TEAA) buffer pH 7.5. 15µl
of 1M silver nitrate solution was added, vortexed, and incubated
at room temperature for 30 mins. This cleaved the
dimethoxytrityl protective group off the thiol, with approx. 70%
efficiency. 20µl of 1.0M dithiothreitol (DTT) was added,
10 vortexed and reacted for 5 mins. The reaction mixture was
centrifuged at 3000 rpm for 5 mins and the supernatant removed.
100µl of TEAA buffer was added to the precipitate, which was
vortexed, centrifuged and the supernatants combined. This oligo
solution was stable for months at -20°C.
15 (ii) Activation of beads and coupling:

Either 0.04 or 0.4 µmole of "Biomag" 4135 thiol-terminated
magnetic affinity chromatography support beads, a product of
Advanced Magnetics Inc., Cambridge, Mass. USA, available in the
UK from Metachem Diagnostics Ltd. of Northampton, (either 1mg,
20 60µl or 10 mg, 0.6 ml.) were washed 3 times with 0.05M TEAA
buffer to remove all mercaptoethanol. The above-prepared oligo
solution (200µl) was added, and vortexed. The solution was
extracted 3 times with ethyl acetate and twice with diethyl ether
to remove all DTT and nitrogen was blown over solution to remove
25 all the solvent. 40 µl of iodine solution (100mg per ml in
glacial acetic acid) was added. The supernatant (minus beads)
should be brown at this stage; if not, iodine solution should be
added in 20µl aliquots until it turns brown. The solution and
beads were then vortexed and reacted for 30 min. to couple the
30 beads to the oligo. After coupling, the beads were centrifuged
and washed three times with 0.05M TEAA buffer to remove iodine
and were then ready for use. To date, 70% of the activated oligo
has been shown to bind to the beads, i.e. a total of 0.02 µmoles
per 1mg or 10 mg of beads. Since the protective group is cleaved
35 with 70% yield and 70% of the activated oligo binds to the beads,

the yield is 70% = 49% with respect to the original oligo.

COUPLING OF AMINE-TERMINATED OLIGONUCLEOTIDE TO BEADS

(a) via cyanogen bromide.

The amino-terminated D99 (4dTTP spaced) oligo (2) prepared
05 above was coupled to Dynabead M450 beads (Dynal UK Ltd) having
–OH active groups according to the method described by V. Lund
et. al., loc. cit. (0.04μ mole DNA to 10 mg of beads).

Beads were activated by incubation for 30 mins. in distilled
water, followed by raising the pH to 11.5 with 2M sodium
10 hydroxide and incubation for 50 mins. 30mg of cyanogen bromide
was added and the pH re-adjusted to 11.5 with 2M sodium
hydroxide. The beads were incubated on a roller for 10 mins
followed by 5 washes with distilled water and resuspended in 10mM
sodium bicarbonate buffer, pH 8.4

15 0.08 μmole of oligo was added to 1ml of 1-methyl imidazole
buffer pH 7 (Sigma) containing 0.1M 1-ethyl-3-(3-dimethyl-
aminopropyl) carbodiimide (Sigma), 0.25M ethylenediamine (Sigma)
and incubated at 50°C for 3 hrs. 60 μl of 3M sodium acetate,
pH6.5 were added and 1 ml ice cold ethanol, mixed and incubated
20 at -80°C for 2 hrs. The precipitate (comprising the amine-
activated oligo), after centrifugation, was dried in a vacuum
dessicator and reconstituted in 600 μl 10 mM sodium bicarbonate
buffer, pH8.0.

The above 600 μl (0.08 μmole) of activated oligo was mixed
25 with 10 mg amine activated beads and incubated for more than 24
hrs on a roller. Control couplings were also performed using
unactivated beads incubated with activated oligo or activated
beads with unactivated oligo.

(b) via carbodiimide

30 Oligo was coupled to M450 beads via carbodiimide according to
the above Lund et al. reference. Thus, 0.08 μmole of activated
oligo was added to 10 mg beads in the presence 0.04M sodium
2-(N-morpholino)ethane sulfonate containing 25 mg 1-cyclohexyl-3-
(2-morpholinoethyl)carbodiimide and incubated overnight at 45°C.
35 Control couplings were performed in the absence of the

carbodiimide reagent.

PREPRARATION OF PROBES

Anti-D99 oligo was 3' end labelled with ^{32}P-dideoxyATP using a kit supplied by Amersham International plc and separated on a
05   G-50 "Sepharose" column. Labelled anti-D99 was added to unlabelled anti-D99 to give a 4-5% label concentration.

HYBRIDISATION OF THE IMMOBILISED OLIGONUCLEOTIDES TO LABELLED PROBES

Beads with or without oligo (0.02 μmole per mg. or 10mg)
10   attached by the methods described above were incubated with a molar excess (0.03 μmole) of anti-D99 (per mg. or 10 mg of beads) (4-5% 3'-end labelled with ^{32}P) in 2 x saline sodium citrate (SSC) buffer at 40°C for 2.5 hrs (1ml total volume). The supernatants were removed and discarded and the beads washed 4
15   times in 2 x SSC at 40°C, to remove thoroughly excess labelled probe.

MEASUREMENT OF AMOUNT OF HYBRIDISATION

The hybridisation capacity of the oligos attached to the beads was then measured by dissociating the labelled probe DNA
20   strand from the bead-bonded oligo strand and measuring the amount of label released into solution. Thus, the beads were then sequentially incubated in 0.5ml of 2 x SSC for 5 mins at 10°C temperature increments up to 90°C, supernatants were removed at the end of each cycle and the number of radioactivity counts per
25   minute (cpm) assessed by Cerenkov-counting. A final further incubation of 90°C in the presence of 2% mercaptoethanol in 2 x SSC was performed and the cpm in the supernatant and remaining on the beads determined.

The results are shown graphically in Figures 1-4 of the
30   drawings in which temperature is plotted on the abscissa and radioactivity released into solution in cpm on the ordinate. At the right-hand side, on a separate abscissa and ordinate the radioactivity remaining on the beads after the final 90°C wash is shown.

35   Figure 1 relates to 10 mg beads dithio-coupled to 0.2 μmole

of oligo D99. The filled squares show the cpm values obtained using the dithio-coupled beads hybridised to labelled complementary oligonucleotide, while the open triangles show the cpm values for the same beads incubated under the hybridisation
05 conditions with the control non-complementary oligonucleotide. The open circles depict the amount of label in the supernatant using unactivated thiol beads (i.e. in the absence of D99) incubated under hybridisation conditions with the complementary (anti-D99) or non-complementary probes (the results were the same
10 whichever probes were used). The right hand side shows the cpm remaining on the beads after the 90°C wash in the presence. 2-mercaptoethanol breaks the -S-S- bond, so these results are a measure of non-specific binding. Similar results (not shown) were obtained in the absence of mercaptoethanol (mean of two
15 experiments).

It will be seen that a satisfactory curve was obtained with the hybrids formed from the dithio-linked beads of the invention. As expected, dissociation of the hybrid strands increased with elevated temperature. The curve is indicative of
20 strong hybridisation.

Figure 2 shows the results obtained from the same experiments performed using 10 mg of beads with or without 0.2 µmole of oligo D99 coupled via the dithio linkage. Filled squares show cpm in supernatants from dithio-linked D99 beads hybridised to labelled
25 complementary (anti-D99), probe whilst open circles represent values obtained using unactivated beads with labelled anti-D99. The results for unactivated beads incubated with non-complementary oligo, and dithio-linked D99 beads incubated with non-complementary oligo are omitted for clarity because they
30 were almost identical to the open circle results, with one exception. The exception was that the dithio-linked D99/non-complementary oligo gave a radioactivity count on the beads between that for dithio-linked D99/non-complementary oligo
35 (filled square) and the unactivated/complementary oligo (open circle).

The results for the dithio-linked beads of the invention were more erratic than in Figure 1, doubtless due to the low ratio of oligo to beads in the coupling reaction.

STABILITY OF LINKAGE

05   It has also been determined that the dithio-linked D99 oligo, either without any nucleotide spacing or with the 4dT spacing as above (0.2μm per 10mg of the above-described beads) withstands pre-incubation for 5 to 30 minutes at temperatures of 60 to 90°C before hybridisation. After carrying out hybridisation and
10   measurement of the amount of hybridisation, as described above, the number of radioactivity counts obtained was satisfactorily high, the maximum being about the same order as that of Figure 1.

COMPARATIVE EXPERIMENTS

Figure 3 shows the results of identical experiments obtained
15   with 10 mg beads coupled to 0.08 micromole of D99 via the cyanogen bromide linkage. Filled circles show cpm in supernatants after hybridisation with an excess of tracer anti-D99 whilst the open symbols show the values obtained using unactivated beads hybridised to labelled anti-D99 or incubated
20   with non-complementary oligo, or coupled beads incubated with labelled non-complementary oligo.

The results in Figures 3 and 4 ought to be multiplied by 2.5 in order to be comparable with Figure 1 (because of the different ratio of beads to oligo in the coupling reaction). However, this
25   would not significantly affect the comparison because the degree of hybridisation of the dithio-linked oligos of the invention is of the order of 100-fold greater (10^5 cpm versus 10^3 cpm).

Figure 4 shows the results obtained as in Figure 3 using beads linked to D99 via carbodiimide chemistry.

30   EXAMPLE 2 (Comparative)

Experiments performed likewise with control pore glass coupling as described for the glass capillary of D99 via cyanogen bromide and carbodiimide show almost identical results to those of Example 1 using those coupling reagents, i.e. as reported in
35   Figures 3 and 4.

EXAMPLE 3

This Example illustrates the effect on hybridisation of including an 8dT spacer arm in the immobilised oligo. Proceeding as in Example 1, with 0.2 µmole of oligo D99 dithio-linked to 10 mg of the magnetic beads (a) with an 8dT spacer arm or (b) for comparison, without a nucleotide spacer arm, the results shown in Figure 5 were obtained. The filled circles represent results from the 8dT spaced immobilised D99, while the filled squares are those for the unspaced immobilised D99. Beads which were not coupled to the D99 gave counts of virtually zero. It is seen that the counts were somewhat higher from the nucleotide spacer arm-linked beads, indicating improved hybridisation.

EXAMPLE 4

This Example illustrates the effect on the hybridisation of including a long (15 dT) spacer arm in the immobilised oligo.

Following the procedure of Example 1, using 10 mg of the beads and, in one run, pre-washing the oligo-coupled beads for 5 minutes at 80°C instead of at room temperature, and using a thiol-terminated oligo with a 15 dT instead of a 4 dT spacer, the data shown in the Table below were generated. There is a reduction in the amount of specific hybridisation compared with Example 1 in which the spacer was shorter. Possibly such a long spacer creates steric hindrance to hybridising anti-D99.

TABLE

Results of hybridisation of labelled anti-D99 to 0.2 µM 15 dT D99 oligo linked to 10 mg magnetic beads. Values represent counts released into supernatant following incubation in 2xSSC buffer at different temperatures shown in the left-hand column; at the bottom the counts from the beads are shown, i.e. analogously to the right-hand side of the Figures.

Wash Temp °C	Control (unactivated Beads)	15dT D99 (room temp pre-wash)	15dT D99 (80°C pre-wash)
40	103853	139028	183856
40	4100	11549	15336
40	1015	3769	1994
40	673	3982	1285
50	1371	7524	5018
60	2452	11495	9587
70	2056	24070	119099
80	4986	21539	10991
90	6152	29617	13471
90 + 2ME	4806	14904	11696
Beads	12499	73681	52337

2ME = 2-mercaptoethanol

EXAMPLE 5

This Example illustrates immobilisation of DNA to a non-thiolated support (control pore glass) by constructing a dithio linkage.

05   0.25 g of 3000 Angstrom CPG, mesh size 60-120 Angstrom were reacted with 0.15ml (3-mercaptopropyl) trimethoxysilane (Aldrich Chemical Co: technical grade 75%) in 3ml of acetone for 5 minutes at room temperature. The CPG was then washed 3 times with acetone, 3 times with diethyl ether and air dried. The yield in

10   this experiment was 8 nanomoles/mg of available thiol groups, as assessed coupling cysteine to the mercaptopropyl-terminated CPG, cleaving the S-S bond, oxidising the cysteine and then amino acid analysis. Taking account of the reduced surface area of CPG compared with the magnetic beads, the yield is some 25-fold less

15   than the thiol groups coupled in previous experiments. Thiol-terminated D99 oligos with 4dT spacer (0.4 μmole) were then added to the CPG (1 mg.) in the way described in Example 1.

The D99 oligos coupled to the activated CPG were then hybridised to labelled anti-D99, as in previous Examples. For

20   comparison, the same hybridisation was performed on 0.2μmole of

D99 coupled to 1mg of magnetic beads as in Example 1.

Since the CPG has 20 fold less thiol groups present on the surface, the hybridisation capacity of this DNA is considered as good as (if not a little better) than that of the magnetic beads.
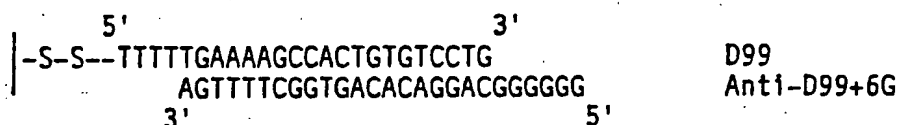
Counts in Supernatants

Wash Temp °C	Control (Unactivated)	Controlled Pore Glass		Magnetic Beads	
		Activated No D99 present	Activated coupled to D99	Activated No D99 present	Activated coupled to D99
40	58256	80682	77450	(61051)	50056
40	4338	3878	4825	(3410)	3438
40	568	377	1084	(1433)	1588
40	160	150	645	(952)	1633
50	124	159	1421	(1669)	2419
60	225	238	5041	(3324)	10701
70	220	314	6205	(4877)	49296
80	394	384	5974	(7747)	102702
90	314	347	705	(4819)	21284
Beads	1134	1250	1358	17074	11282

05 EXAMPLE 6

This following Example demonstrates that double-stranded DNA simulating a PCR product, immobilised onto magnetic particles (Biomag) via the dithio-linkage described in Example 1, can be labelled by a 3' end-labelling reaction.

10 3 mg magnetic beads having oligo D99 with a 4 dT spacer (0.2 μM per 10mg beads) coupled via the dithio-linkage of the invention were incubated for 1 hour at 40°C with an excess of complementary oligo (anti-D99) either (1) as such or (2) with an additional 5'-poly dG sequence (of 6 dG bases) extending past the 15 free 3' terminus of the immobilised oligo D99, (as shown below):

```
        5'                          3'
|-S-S--TTTTTGAAAAGCCACTGTGTCCTG              D99
|       AGTTTTCGGTGACACAGGACGGGGGG           Anti-D99+6G
        3'                          5'
```

The magnetic beads carrying this duplex of immobilised

double-stranded DNA were used as template in the 3' end-labelling
reaction employing ^{32}P-dCTP as the labelling nucleotide, and Taq
polymerase or Klenow fragment as the enzymes in Taq polymerase
buffer. After labelling, whereby cytidine (dC) bases are added
05   to complement the guanosine (dG) tail, the reaction mixture was
heated to 90°C, and whilst still at this temperature, the beads
were removed by centrifugation. The beads were washed with 2xSSC
at 90°C and the radioactivity (cpm) remaining on them was then
determined by Cerenkov-counting. The results (Table 1) were
10   compared to those obtained using D99-beads without enzyme
present, or using magnetic beads alone (i.e. no immobilised D99).

The results demonstrate that substantial radioactive label
(and therefore incorporation of ^{32}P-dCTP to the 3' terminus of
oligo D99) is present only using magnetic beads with
15   dithio-linked oligo D99 in the presence of either Taq polymerase
or Klenow fragment. Thus, the 3' terminus of the dithio-linked
oligo D99 on the magnetic beads is accessible to the DNA
polymerase activity of both enzymes under the appropriate
conditions. When Klenow fragment was used as the enzyme for the
20   labelling reaction, the beads having the D99/anti-D99 duplex
without the dG tail showed a high "background" radioactive
count. This can be ascribed to intrinsic exonuclease activity of
Klenow fragment.

TABLE 1

25   Amount of radioactivity (cpm) on beads after being subjected
to 3' end-labelling reaction using ^{32}P-dCTP

Enzyme	D99 Beads + anti-D99+6G	D99 Beads + anti-D99 (control)	Plain Beads (no D99)
None	627	2004	774
Klenow	388359	94225	11093
Taq polymerase	1137481	8598	921

EXAMPLE 7

This Example demonstrates that DNA (oligo D99) dithio-linked to magnetic beads can function as the forward primer in the polymerase chain reaction for the amplification of target DNA
05 (the E6 region of human papilloma virus type 16 (HPV16) genomic DNA), resulting in immobilisation of the PCR products as double stranded or (by heating the product to 90°C immediately before separating solid from liquid phases) single stranded DNA.

3 mg magnetic beads coupled to oligo D99 (as in the previous
10 example) were added to a total 100 µl PCR reaction mixture composed of recommended PCR buffer (Perkin-Elmer Cetus) in the presence of Caski DNA (a cell line infected with HPV16 genomic DNA) free (i.e. not immobilised) reverse primer (referred to as non-complementary primer in Example 1) or both free
15 non-complementary primer and oligo D99. Reactants were preheated to 98°C for 7 minutes 5 units of Taq polymerase were added and a PCR was carried out for 40 temperature cycles composed of 96°C for 30 seconds (denaturation), 40°C for 30 seconds (annealing of primer to target) and 72°C for 1 minute (DNA synthesis by primer
20 extension) performed on a Wolfson Programmable Thermal Cycler. Following cycling, supernatants were removed and the beads resuspended in 2xSSC heated to 90°C. The magnetic beads were removed by centrifugation and washed once with 2xSSC buffer at room temperature. This results in the dithio-linked oligo D99
25 and its extension product remaining on the magnetic beads as single-stranded DNA. The beads were then probed with a [32]p hexanucleotide-labelled 38mer oligo complementary to a mid-sequence of the expected HPV16 PCR product (probe sequence = 5' AAAGATTCCATAATATAAGGGGCGGTGACCGGTCGATG 3', identified as
30 Sequence ID No. 4). Hybridisation of pobe and assessment of hybridised probe were performed as described in Example 1, except that the amount of radioactivity remaining on the beads was determined. The results are given in Table 2 which also includes the data obtained using magnetic beads alone (i.e. no
35 dithio-linked oligo D99) in the presence or absence of Taq polymerase and in reaction mixtures containing

free (i.e. unlinked) reverse primer and forward primer.

TABLE 2

Results of hybridising ^{32}P-labelled mid-sequence probe to oligo D99 coupled magnetic beads after PCR cycling. Numbers refer to cpm after washes at different temperatures.

Bead Type	Free Oligo	Taq	40°C	50°C	60°C	70°C	80°C	90°C
no D99	F + R	+	13748	4948	5057	5080	7487	3783
+ D99	R alone	+	80287	77817	54634	19681	13724	9993
+ D99	F + R	+	66574	67716	61856	38463	34809	19369
no D99	F + R	−	4123	3129	2873	3770	4399	3084
+ D99	R alone	−	23069	23908	18456	16807	17337	11923
+ D99	F + R	−	22437	20035	18977	15365	13694	12813

F refers to forward oligo primer (i.e. D99) and R refers to reverse primer. "Taq" = Taq polymerase.

The results demonstrate that using dithio-linked D99 magnetic particles, in the presence of free reverse primer (or both primers free) and Taq polymerase, specific amplification of the HPV16 E6 region is permissible.

The reaction scheme below shows a simplified version of a PCR carried out in the manner of Example 7, with a forward primer (D99) immobilised, a reverse primer in solution, and a single molecule of double-stranded HPV16 DNA.

If one amplification cycle is performed on dsDNA of HPV16 consisting of strands labelled "B" and "C" the results would be:

(1) Extension of the immobilised forward primer to yield one new HPV16 strand of DNA immobilised to the solid phase, labelled "A".

(2) Extension of the reverse primer in solution to yield one new strand of HPV16 DNA which is opposite in sequence and orientation (i.e. complementary) to the strand generated in (1) above. This new strand is labelled "D".

(3) The two separate strands "B" and "C", of the original HPV16 DNA.

Upon cooling the reaction mixture to allow re-annealing of each of the separate strands "A", "B", "C" and "D", the following immobilised products would be obtained:
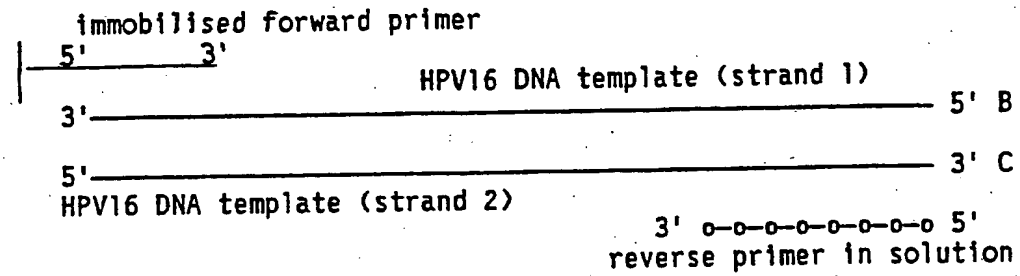
(4) immobilised dsDNA composed of the new strand "A" formed
05   by forward primer annealed to complementary strand "B" of HPV16 starting DNA template; or

(5) immobilised dsDNA composed of the new strand "A" formed by the forward primer annealed to the new complementary strand "D" generated by the reverse primer.

10   Both situations essentially result in one dsDNA immobilised and one in free solution. Further amplification in the presence of more of each primer, then (as in a regular PCR reaction) results in more dsDNA product (original template cannot increase) which is immobilised to the solid phase. By heating to 90-100°C,
15   the dsDNA is made single stranded which, using the above conditions, results in all the forward primer product being immobilised and all the reverse primer product being in solution.

FIRST CYCLE

STAGE 1 : ANNEALING OF PRIMERS

immobilised forward primer

```
| 5'_____3'                        HPV16 DNA template (strand 1)
|  3'_____ 5' B

   5'_____ 3' C
   HPV16 DNA template (strand 2)
                                        3' o—o—o—o—o—o—o—o 5'
                                        reverse primer in solution
```
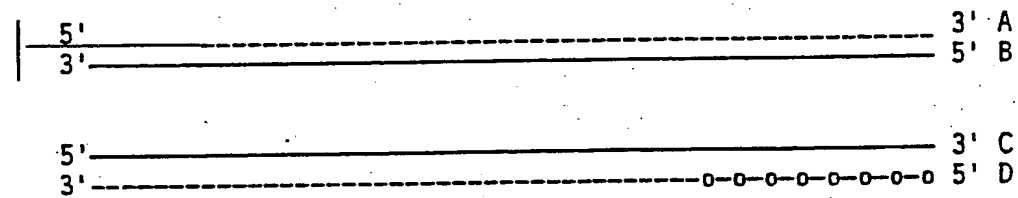
STAGE 2 : PRIMER EXTENSION, FOLLOWED BY SEPARATING THE STRANDS

```
| 5'_____ 3' A
|  3'_____ 5' B

   5'_____ 3' C

   3'_____o—o—o—o—o—o—o—o 5' D
```
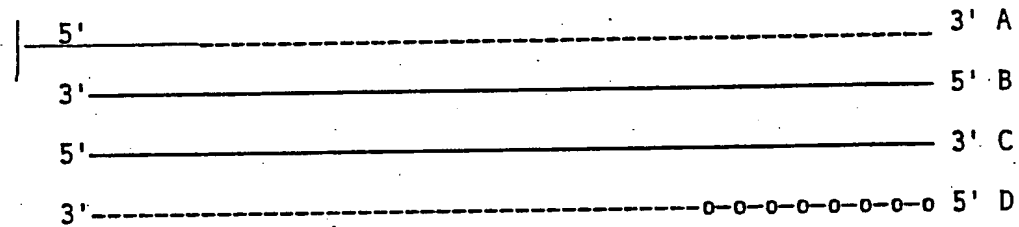
RE-ANNEALING OF THE SINGLE STRAND PRODUCTS

```
| 5'_____ 3' A
|  3'_____ 5' B


   5'_____ 3' C
   3'_____o—o—o—o—o—o—o—o 5' D
```

or

```
| 5'_____ 3' A
|  3'_____o—o—o—o—o—o—o—o 5' D


   3'_____ 5' B
   5'_____ 3' C
```
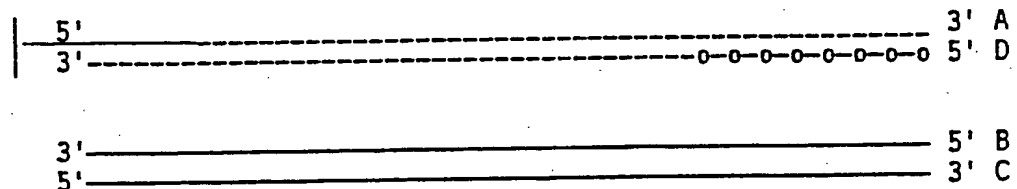
Of course, if the reverse primer were also to be immobilised, then all the solid phase products would contain either the target HPV16 DNA (one strand or the other) or a copy of part of the target DNA made by the primer extension.

SEQUENCE LISTING

This sequence listing is provided in this International Patent Application to meet the requirements or wishes of certain contracting States (EPC countries, US, JP). The "General Information Section" is applicable only to US.

(1) GENERAL INFORMATION:

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                     WALKER, Matthew R.

    (ii) Title of Invention :   Immobilised Polynucleotides

    (iii) Number of Sequences :   4

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                                    U.S.A. 22201

    (v) Computer Readable Form:

        (a) Medium Type   : Diskette, 5.25 inch, 360 Kb storage.
        (b) Computer   : IBM PC/AT compatbible.
        (c) Operating System: MS-DOS 3.2.
        (d) Software   : Word Perfect ASCII File Format.

    (vi) Current Application Data:

              (a) Application Number :
              (b) Filing Date:
              (c) Classification:

    (vii) Prior Application Data:

              (a) Application Number : PCT/GB 90/
              (b) Filing Date   :

              (a) Application Number : GB 8915607.9
              (b) Filing Date   : 07 JUL 1989

              (a) Application Number : GB 8921327.6
              (b) Filing Date   : 21 SEP 1989

              (a) Application Number : GB 9008274.4
              (b) Filing Date   : 11 APR 1990

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(2) INFORMATION FOR SEQUENCE ID NO. 1
    (i) Sequence Characteristics :
        (a) Length   : 20 base pairs
        (b) Type   : Nucleic acid
        (c) Strandedness : Double
        (d) Topology   : Linear
    (xi) Sequence Description : Sequence ID No. 1
        TCAAAAGCCA CTGTGTCCTG   20

(2) INFORMATION FOR SEQUENCE ID NO. 2
    (i) Sequence Characteristics:
        (a) Length   : 20 bases
        (b) Type   : Nucleic acid
        (c) Strandedness : Single
        (d) Topology   : Linear
    (xi) Sequence Description : Sequence ID No. 2
        CAGGACACAC TGGCTTTTGA   20

(2) INFORMATION FOR SEQUENCE ID NO. 3
    (i) Sequence Characteristics:
        (a) Length   : 20 bases
        (b) Type   : Nucleic acid
        (c) Strandedness : Single
        (d) Topology   : Linear
    (xi) Sequence Description : Sequence ID No. 3
        CGTGTTCTTG ATGATCTGCA   20

(2) INFORMATION FOR SEQUENCE ID NO. 4
    (i) Sequence Characteristics
   (a) Length   : 38 bases
        (b) Type   : Nucleic acid
        (c) Strandedness : Single
        (d) Topology   : Linear
    (xi) Sequence Description : Sequence ID No. 4
        AAAGATTCCA TAATATAAGG GGCGGTGACC GGTCGATG   38

CLAIMS

1. An immobilised polynucleotide material comprising a support bound to a 5'-end of at least one polynucleotide through a linkage which comprises a dithio (-S-S-) group.

2. An immobilised polynucleotide material according to Claim 1
05 wherein the polynucleotide contains a "c-target" base sequence complementary to a base sequence of interest in a target polynucleotide, and wherein the "c-target" sequence is spaced from the support so as to permit the c-target sequence to hybridise to the target polynucleotide.

10 3. An immobilised polynucleotide material according to Claim 2 wherein the c-target sequence is spaced from the 5'-end of the polynucleotide by a spacer arm of one or more nucleotides which do not participate in or interfere with c-target/target hybridisation.

15 4. An immobilised polynucleotide material according to Claim 3 wherein the nucleotide spacer arm is from 3 to 20 nucleotides long.

5. An immobilised polynucleotide material according to Claim 4 wherein the nucleotide spacer arm is from 4 to 15 nucleotides
20 long.

6. An immobilised polynucleotide material according to any preceding claim wherein the linkage includes a spacer arm between the support and the -S-S- group, between the -S-S- group and the 5'-end of the polynucleotide or both.

25 7. An immobilised polynucleotide according to Claim 2 comprising a nucleotide spacer arm according to Claim 3, 4 or 5 and a linkage spacer arm according to Claim 6, which together conform to the general formula:

$$(N)_x - (L_1)_y\text{-S-S-}(L_2)_z$$
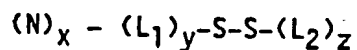
30 where:

N is a nucleotide attached to the 5'-end of the c-target sequence and where there is more than one such nucleotide they may be the same or different;

L_1 and L_2 are atoms in a chain from the 5'-end nucleotide of the polynucleotide to the support, on each side of the two S atoms of the -S-S-linkage;

x, y and z are 0 or integers and the sum of x, y and z is 3 to 25.

8. A method of producing an immobilised polynucleotide material according to Claim 1, which comprises reacting a 5'-(thiol-terminated) derivative of at least one polynucleotide with a support having a thiol group thereon, either or both of said thiol groups being -SH groups as such or derivatised as co-reactive -S-S- linkage-producing derivatives, to form a said -S-S- linkage between said thiol groups.

9. A method according to Claim 8, wherein the polynucleotide contains a "c-target" base sequence complementary to a target sequence of interest in another polynucleotide and wherein spacing is provided within the thiol-terminated polynucleotide between the target sequence and the thiol termination, within the thiolated support between the support and the thiol group or within both, said spacing being in total, after reaction to form the -S-S- linkage, effective to space the support from the c-target sequence to permit the c-target sequence to hybridise to the target sequence.

10. A method according to Claim 9 wherein spacing is provided as defined in Claim 4, 5 or 7.

11. A method of carrying out a polymerase chain reaction (PCR) in which an immobilised polynucleotide material claimed in any one of Claims 1 - 7 or prepared by a method claimed in any one of Claims 8 - 10 is substituted for either or both of the oligonucleotide primers used in the PCR and the amplified product of the PCR is separated on the solid phase.

12. A method of carrying out a PCR reaction which comprises:

(1) annealing forward and reverse oligonucleotide primers, which are immobilised polynucleotide materials as defined in Claim 11, to the strands of a polynucleotide to be amplified which serve as

templates, the annealing being carried out at a temperature of 30 to 60°C.

(2) extending the primed sequences along their respective template strands of the polynucleotide to be amplified, at a temperature of from 60 to 80°C,

(3) separating the thus synthesised strands from their templates at a temperature of 80 to 100°C and repeating the process by using the separated strands as templates.

13. A method according to Claim 11 or 12 wherein the amplified products of the PCR are labelled and the PCR is used to assay a sequence of the polynucleotide to be amplified.

14. A method of heterogeneous assay for an analyte nucleic acid, which comprises subjecting an immobilised polynucleotide material according to any one of Claims 1 - 7 or prepared by a method claimed in any one of Claims 8 - 10, having a "c-target" base sequence complementary to a target sequence of analyte nucleic acid present in a liquid phase analyte, hybridising the c-target sequence to the target sequence separating the phases and determining the occurrence or extent of hybridisation.

15. A method according to Claim 14 wherein the analyte nucleic acid is generated by a liquid phase PCR.